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The Cbl Family and Other Ubiquitin Ligases: Destructive Forces in Control of Antigen Receptor Signaling

Review

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Summary

Regulation of tyrosine kinase-mediated cellular activation through antigen receptors is of great biological and practical significance. The evolutionarily conserved Cbl family ubiquitin ligases have emerged as key negative regulators of activated tyrosine kinase-coupled receptors, and their impaired function switches a normal immune response into autoimmunity. Cbl proteins facilitate the ubiquitinylation of activated tyrosine kinases and other signaling proteins and of the signaling chains of receptors themselves; monoubiquitin tag promotes sorting of activated receptors and associated proteins into internal vesicles of the multivesicular body, facilitating their lysosomal degradation, whereas polyubiquitin tag promotes proteasomal degradation. Notably, increased expression of Cbl proteins and other ubiquitin ligases is a component of anergic signaling program in T cells. Thus, controlled destruction of the signaling apparatus has emerged as a key to fine-tuning antigen receptor signaling. Further studies of this pathway are likely to elucidate the pathogenesis of autoimmune diseases and offer new therapeutic targets.

Introduction

Appropriate triggering of lymphocyte antigen receptors (T cell receptor [TCR] and B cell receptor [BCR]) by self- and foreign antigens is crucial for the development of a mammalian immune system geared to protection of the body against pathogens and transformed cells without the induction of autoimmunity or serious inflammatory diseases. Recent advances in cellular immunology have elegantly demonstrated that the strength and duration of lymphocyte activation are crucial in determining the fate of responding lymphocytes. Thus, strongly stimulating self-antigens can delete autoreactive lymphocytes during development or through peripheral tolerance (Goodnow et al., 2001; Love and Chan, 2003). Conversely, antigen receptor stimulation in the absence of costimulatory molecules or stimulation with weak antigens elicits a program of anergy (Appleman and Boussiotis, 2003; Schwartz, 2003). Elucidation of the biochemical mechanisms that control the duration and intensity of antigen receptor signals, and consequently determine the activation, deletion, or anergic fate of lymphocytes upon antigen encounter, therefore represents a major challenge for immunologists and cell biologists.

Activation of protein tyrosine kinases represents the most proximal biochemical event in antigen receptor-mediated lymphocyte activation (Kane et al., 2000; Qian and Weiss, 1997; van Leeuwen and Samelson, 1999). Activation of membrane-anchored Src family kinases (such as Lck and Fyn) upon receptor and coreceptor clustering leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs within the cytoplasmic tails of the receptor signaling chains, resulting in the recruitment and Src family kinase-mediated activation of cytoplasmic Syk/ZAP70 kinases. These two families of tyrosine kinases, together with members of the Tek kinase family (such as ITK and BTK), are crucial for phosphorylation-mediated assembly of signaling complexes and activation of a number of biochemical pathways. Key pathways that have been established to contribute to lymphocyte activation include the PLC-γ-mediated calcium flux, which in turn activates NFAT-mediated transcription; protein kinase C activation; Ras-MAP kinase-mediated activation of transcription through AP-1, and cell cycle progression; phosphoinositide (PI) 3-kinase-mediated costimulatory and survival signals; and Rho/Rac/cdc42- and Rap-mediated cytoskeletal rearrangements, integrin activation, and migratory effects (Figure 3). There is extensive crosstalk among the components of these and other signaling pathways, which presumably promotes signal specificity and fine-tunes the cellular responses. Recent studies, in particular in T lymphocytes, have demonstrated that many of the tyrosine kinase-mediated signaling events are orchestrated within the immunological synapse, the area of specialized cell-cell contact between a lymphocyte and the antigen-presenting cells (APC) (Bromley et al., 2001). Thus, it is beginning to be appreciated that the molecular mechanisms that control the duration and intensity of lymphocyte antigen receptor signaling are closely linked with those that control the spatio-temporal organization of antigen receptors, coreceptors, costimulatory molecules, and signaling proteins within the immunological synapse (Lee et al., 2003).

The versatility of tyrosine phosphorylation as a signaling switch reflects its reversibility by the removal of phosphate moiety through the action of phosphotyrosine phopsphatases. Indeed, this class of enzymes plays a crucial role in setting the threshold of antigen receptor signals (Hermiston et al., 2003; Mustelin and Tasken, 2003). Recent studies have, however, identified a new class of negative regulators that control the intensity and duration of receptor-generated signals by specific ubiquitin modification of the activated antigen receptors, associated tyrosine kinases, and downstream signaling proteins (Ben-Neriah, 2002; Dikic et al., 2003; Jang and Gu, 2003; Jun and Goodnow, 2003; Krawczyk and Penninger, 2001; Latour and Veillette, 2001; Lupher et al., 1999; Miyake et al., 1997; Rao et al., 2002a; Rudd and Schneider, 2000; Sawasdikosol et al., 2000; Thien

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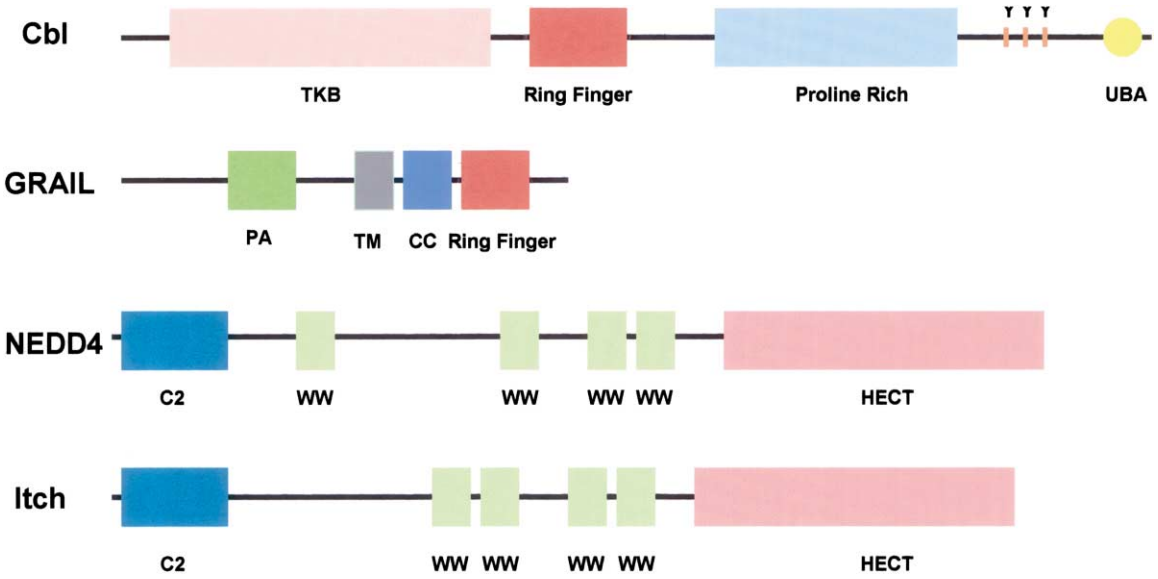

Figure 1. Schematic Structure of Various Ubiquitin Ligases Involved in the Regulation of Antigen Receptor Signaling, Autoimmunity, and Anergy

Shown are the RING finger domain-containing ubiquitin ligases Cbl (Cbl-b has an essentially identical architecture) and GRAIL, and the HECT domain-containing ubiquitin ligases of the NEDD4 family, NEDD4 and Itch. The domain acronyms are: TKB, tyrosine kinase binding; RING (really interesting new gene); UBA, ubiquitin-associated; PA, protease-associated; TM, transmembrane; CC, coiled-coil; C2, calcium binding C2 domain; WW, two tryptophan(W)-containing domain (binds proline-rich sequences); HECT, homologous to E6-associated protein C terminus. Y in Cbl represent the conserved sites of tyrosine phosphorylation (Y700, 731, and 774 in human Cbl).

and Langdon, 2001; Tsygankov et al., 2001; Weissman, 2001). This new mechanism, mediated by the ubiquitin ligases of the Cbl family as well as other recently identified ubiquitin ligases, is the focus of this review.

Cbl Family Proteins—Ubiquitin Ligases for Activated Tyrosine Kinases

Three distinct Cbl (for Casitas B lymphoma) proteins are found in mammals, with Cbl and Cbl-b being highly related in structure, while Cbl-c lacks some of the C-terminal motifs (Figure 1) (Dikic et al., 2003; Lupher et al., 1999; Miyake et al., 1997; Rao et al., 2002a; Sawasdikosol et al., 2000; Thien and Langdon, 2001; Tsygankov et al., 2001; Weissman, 2001). The signature motifs of this protein family, from *C. elegans* to man, are two highly conserved N-terminal domains: the tyrosine kinase binding (TKB) domain, itself composed of a four-helix bundle, a calcium binding EF hand, and an incomplete SH2 domain, mediates binding to specific phosphotyrosine motifs on activated PTKs and possibly other signaling proteins; and a RING finger domain that interacts with ubiquitin-conjugating enzymes (E2s). These two domains together define the basic functional unit of Cbl proteins, a ubiquitin ligase directed at activated tyrosine kinases (Figure 2). In this scheme, the TKB domain binding to a consensus phosphotyrosine motif (typically N/DXpYXXXϕ; where ϕ is any hydrophobic residue, usually proline) (Levkowitz et al., 1999; Lupher et al., 1997; Meng et al., 1999; Peschard and Park, 2003), generated as a result of autophosphorylation (or transphosphorylation), serves to dock Cbl proteins on an active tyrosine kinase. The RING finger-associated E2 then transfers ubiquitin, received from E1 (ubiquitin-activating enzyme), to the tyrosine kinase substrate. The fate of the ubiquitinylated substrates varies and is fully discussed below.

Certain Cbl family members, such the highly homologous mammalian Cbl and Cbl-b, contain additional structural motifs that allow physical association with additional components of the tyrosine kinase signaling pathways (Figure 3) (Dikic et al., 2003; Liu, 2004; Lupher et al., 1999; Miyake et al., 1997; Rao et al., 2002a; Sawasdikosol et al., 2000; Thien and Langdon, 2001; Tsygankov et al., 2001; Weissman, 2001). Most of these interactions occur following receptor triggering. Three major tyrosine phosphorylation sites on Cbl, generated upon activation through a number of receptors, serve as SH2 domain docking sites and mediate association with PI 3-kinase (via its p85 regulatory subunit), Crk family of adaptor proteins, Vav family of Rho/Rac guanine nucleotide exchange factors, and possibly Src family kinases. A proline-rich region, which is relatively extensive in Cbl and Cbl-b, allows binding to a number of SH3 domain-containing signaling proteins; in the context of antigen receptor signaling, the most prominent among these are Src family kinases, and adaptor proteins such as Grb2 and Nck, as well as the p85 subunit of PI 3-kinase and the Vav guanine nucleotide exchange factor. While the SH3 domain proline-rich sequence interactions do not require a posttranslational modification and could occur in resting cells, the SH3 domains in signaling proteins are often unavailable prior to receptor activation, as best exemplified by Src family kinases (Xu et al., 1999). Overall, many of the protein-protein interaction motifs in Cbl proteins are likely to serve the basic function of juxtaposing substrates next to a RING finger-associated E2, although a ubiquitin ligase-independent adaptor role of Cbl still remains a possibility. A ubiquitin-associated

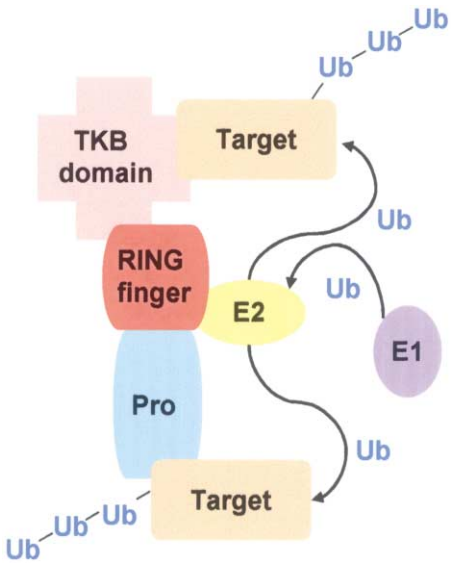

Figure 2. Model of Cbl Ubiquitin Ligase Function

A ubiquitin-activating enzyme (E1, purple) activates Ub and transfers it to a ubiquitin-conjugating enzyme (E2, yellow) which interacts with a ubiquitin ligase (E3) and transfers Ub to the target protein (peach). The RING finger mediates binding to the E2. Multiple motifs in Cbl proteins, such as the tyrosine kinase binding (TKB, pink) domain, the proline-rich region (blue), or the phosphorylated tyrosine residues (not shown) serve to recruit the substrates for ubiquitinylation. The induced ubiquitinylation may be in the form of monoubiquitin units or polyubiquitin chains, leading to lysosomal or proteasomal targeting, respectively.

(UBA) domain near the C terminus of Cbl and Cbl-b can mediate ubiquitin binding and dimerization; however, the functional roles of this region are less clear at present (Bartkiewicz et al., 1999).

Critical Role of Cbl Proteins as Negative Regulators of Antigen Receptor Signaling

Genetic studies have conclusively confirmed the crucial role of Cbl proteins as negative regulators of antigen receptor signaling that was initially deduced from cellular and biochemical studies (see below). Two sets of Cbl as well as Cbl-b knockout mice have been generated, and these have revealed distinct phenotypes of Cbl and Cbl-b deficiencies (Bachmaier et al., 2000; Chiang et al., 2000; Murphy et al., 1998; Naramura et al., 1998). Cbl$^{-/-}$ mice generated by the Bowtell laboratory exhibited increased cell numbers in the thymus, spleen, and lymph nodes, as well as increased circulating platelet numbers and enhanced ductal density and branching in the mammary gland (Murphy et al., 1998). However, Cbl-deficient mice generated in the Gu laboratory did not appear to have a change in thymocytes numbers, although the phenotypes of other organs were not reported (Naramura et al., 1998). Notably, crossing the latter mice with TCR transgenic lines revealed that Cbl deficiency enhanced the positive selection of CD4^{+} but not the CD8^{+} thymocytes. In both sets of Cbl$^{-/-}$ mice, CD4/8 double-positive (DP) thymocytes showed higher levels of TCRβ and CD3 on the cell surface, possibly reflecting enhanced positive selection. Finally, while the thymocytes from Cbl$^{-/-}$ mice showed increased proliferation upon anti-CD3 stimulation (Murphy et al., 1998), peripheral T cells showed reduced proliferation (Naramura et al., 1998), presumably because the enhanced positive selection in these mice allows low responder T cells that would otherwise die of neglect to be positively selected and exported to the periphery.

In contrast to the Cbl$^{-/-}$ mice, Cbl-b$^{-/-}$ mice generated in two different laboratories showed no obvious developmental abnormalities in the immune system or elsewhere (Bachmaier et al., 2000; Chiang et al., 2000). Yet, these mice succumbed to widespread autoimmune disease and widespread inflammatory tissue damage, either when immunized with myelin basic protein (Chiang et al., 2000) or spontaneously (Bachmaier et al., 2000). Analysis of the peripheral lymphoid compartment of these mice revealed dramatic elevation in proliferation and cytokine production by T lymphocytes in response to antigen or anti-TCR antibodies and enhanced cytolytic activity of CD8^{+} cells. Remarkably, Cbl-b-deficient T cells did not require CD28 costimulation for proliferative or cytokine responses to receptor triggering. Furthermore, Cbl-b deficiency was able to rescue the hyporesponsiveness of CD28 and Vav knockout mouse T cells to TCR triggering (Krawczyk et al., 2000). The presence of increased anti-DNA IgM antibodies and hyperreactivity of B cells (Bachmaier et al., 2000) in Cbl-b knockout mice indicates that the hyperreactive phenotype extends to both T and B lymphocytes. Thus, while strain differences, environmental conditions, or the knockout strategy used may have contributed to some differences in the knockout phenotypes, it is clear that single knockouts of Cbl and Cbl-b genes lead to distinct yet marked lymphoid hyperresponsive phenotypes. The predominantly thymic versus peripheral lymphoid effects of Cbl and Cbl-b deficiency correlate closely with their relative levels in the thymus versus the peripheral T cells (Naramura et al., 2002).

The insights provided by Cbl and Cbl-b single knockout mice have been further boosted by recent analyses of Cbl/Cbl-b double knockout mice. Given the relatively narrow pattern of Cbl-c expression, primarily in epithelial tissues (Griffiths et al., 2003), it is not surprising that Cbl and Cbl-b double knockout mice show embryonic lethality prior to gestational day 10 (Naramura et al., 2002). This observation shows an essential and redundant role of Cbl and Cbl-b during development, as would be expected from the wide range of the receptor tyrosine kinases and other signaling proteins aside from lymphoid tyrosine kinases that are negatively regulated by Cbl proteins (Davis and Ben-Neriah, 2004; Liu, 2004; Lupher et al., 1999; Miyake et al., 1997; Rao et al., 2002a; Sawasdikosol et al., 2000; Thien and Langdon, 2001; Tsygankov et al., 2001; Weissman, 2001). Naramura et al. therefore generated Lck-Cre^{+}/Cbl$^{flox/flox}$/Cbl-b$^{-/-}$ mice, with a conditional double deletion of Cbl and Cbl-b in T cells (Naramura et al., 2002). These mice developed widespread autoimmune tissue damage within a few months leading to early death, despite being housed in a pathogen-free environment. Peripheral T cells from these mice were predominantly of a memory phenotype, compared to either the wild-type mice or the single knockouts. The T cell hyperproliferation and increased cytokine production in these mice was dramatically more than that seen

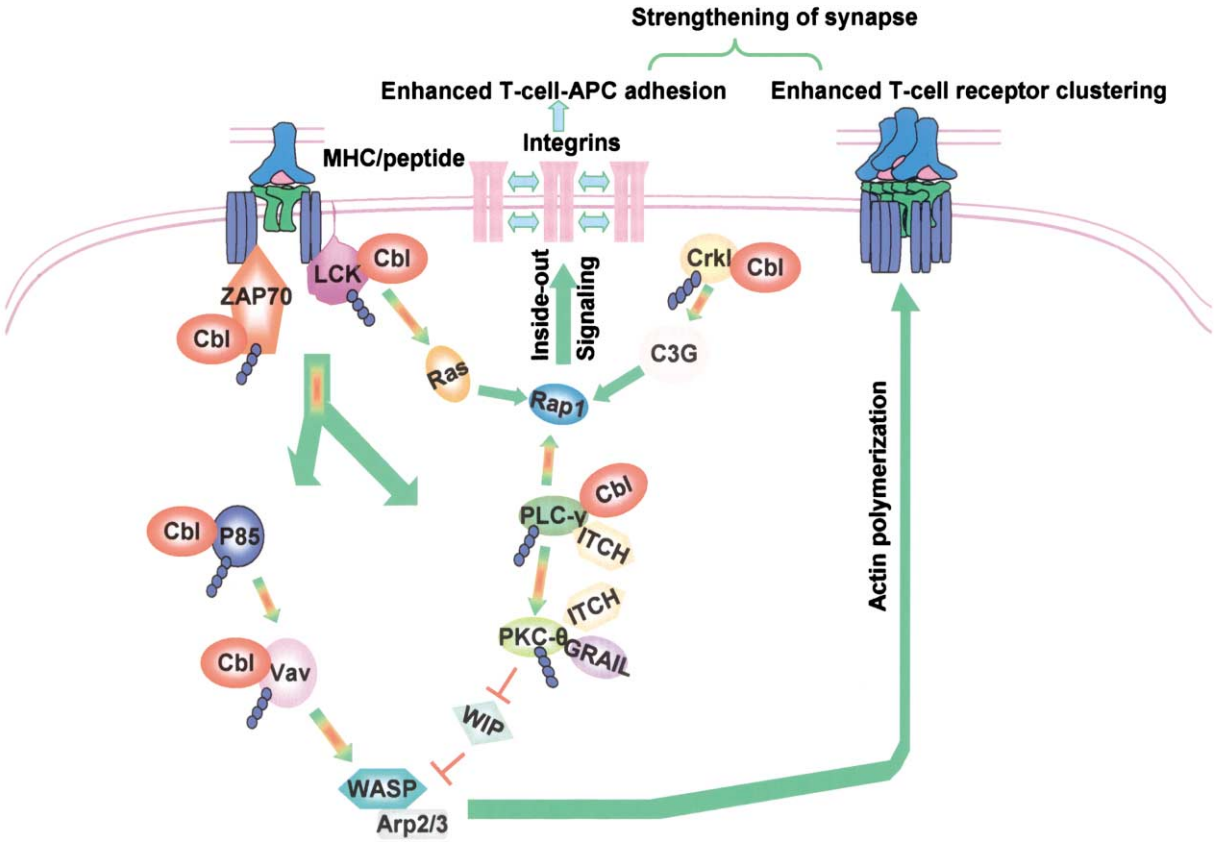

Figure 3. Model of Negative Regulation of T Cell Receptor Signaling and Induction of Anergy through the Ubiquitinylation of Tyrosine Kinases and Downstream Signaling Proteins

Ubiquitinylation of proximal tyrosine kinases, such as ZAP70 and Lck (or other Src family kinases), targets them for degradation; the resulting reduction in the phosphorylation of effector proteins decreases the intensity and duration of multiple downstream signals. Direct ubiquitinylation of downstream effectors leads to negative regulation of these signaling pathways through degradation or degradation-independent regulatory effects; these lead to reduced calcium flux (inhibition of PLCγ), reduced actin polymerization and TCR clustering (via Vav), and inhibition of inside-out signaling to integrins followed by weakening of the immunological synapse (via inhibition of Rap1). Induction of Cbl family and other ubiquitin ligases by anergy-inducing stimuli accentuates these mechanisms, leading to the abrogation of the T cell activation program and destabilization of the immunological synapse induced by binding to the APC-presented MHC/peptide. Cbl refers to both Cbl and Cbl-b. WASP, Wiskott-Aldrich-syndrome protein; WIP, WASP-interacting protein; Arp, actin-related protein. Ubiquitin ligases function in an analogous fashion in the context of other antigen receptors. The red color within the green arrows indicates the steps within the signaling pathway that are attenuated by ubiquitinylation.

in Cbl-b knockout T cells. Thus, Cbl and Cbl-b together appear to be essential mediators of a negative regulatory pathway that serves the physiological role to attenuate lymphocyte activation and to prevent autoimmunity.

Complementing the insights provided by Cbl and Cbl-b knockout mice, studies in the Komeda Diabetes-Prone (KDP) rat model have provided further genetic evidence for the role of Cbl-b in attenuating lymphocyte reactivity (Yokoi et al., 2002). Nearly 100% of KDP rats develop insulitis by day 220 of age, and almost 80% show frank type I diabetes. Positional cloning identified the mutant gene to be Cbl-b: a nonsense mutation at codon 455 was observed, which is predicted to result in a truncated protein with the TKB and RING finger domains but lacks the remaining C-terminal sequences. Transgenic rescue of Cbl-b expression prevented insulitis and diabetes, establishing Cbl-b as an autoimmune susceptibility gene. The predominance of the autoimmune manifestations in the pancreas of KDP rat, as opposed to more general inflammatory manifestations in Cbl-b knockout or Cbl/Cbl-b double knockout mice, is likely to reflect the postulated presentation of a β cell antigen by the MHC class II "u" proteins of this strain to T cells (Yokoi et al., 2002).

Thus, initial genetic studies in mice and rats provide strong support for the role of Cbl family proteins as crucial determinants of a self-limiting versus an uncontrolled immune response with autoimmune consequences if Cbl protein function, especially that of Cbl-b, is compromised in mature T cells. Notably, the lack of any autoimmune phenomena in Cbl knockout mice may reflect the elimination of potentially autoreactive lymphocytes during development, although further studies are required to fully assess the nature of immunological defects in these mice.

Biochemical and Cell Biological Basis of Negative Regulation by Cbl Ubiquitin Ligases

Ubiquitinylation and Degradation of Antigen Receptor-Associated Tyrosine Kinases

Both families of tyrosine kinases that are critical for proximal antigen receptor signaling, the Syk/ZAP70 and

Src family kinases, are targeted by Cbl proteins. The negative regulatory autophosphorylation sites within the SH2 domain kinase linker of ZAP70 (Y292) or Syk (Y323) mediate Cbl protein recruitment to activated kinases by serving as docking sites for the TKB domain. Transfection analyses in model cells as well as T cell or B cell lines have demonstrated that association with Cbl leads to the ubiquitinylation and subsequent degradation of ZAP70 or Syk, and inhibition of cellular responses such as CD3-induced IL2 gene transcription in T cells (Deckert et al., 1998; Lupher et al., 1996, 1997, 1998; Meng et al., 1999; Ota et al., 2000; Rao et al., 2000, 2001). Consistent with this mechanism, anti-CD3 stimulated thymocytes from Cbl$^{-/-}$ mice show more persistent ZAP70 phosphorylation, and B cells from Cbl-b knockout mice exhibit prolonged anti-IgM-induced Syk phosphorylation (Murphy et al., 1998; Sohn et al., 2003). Notably, the Gu as well as the Penninger laboratories reported that TCR-induced ZAP70 phosphorylation was unchanged in Cbl-b knockout T cells, although some elevation was apparent in the Penninger study (Bachmaier et al., 2000; Chiang et al., 2000); in contrast, the Cbl/Cbl-b double knockout peripheral T cells showed a marked elevation of ZAP70 phosphorylation (Naramura et al., 2002). Although mast cell signaling in Cbl/Cbl-b knockout mice remains to be analyzed, transfection studies have demonstrated the Cbl-mediated negative regulation of Syk and Fcϵ receptor signaling (Ota and Samelson, 1997; Qu et al., 2004). Thus, there is strong evidence that one critical mechanism for the negative regulatory role of Cbl proteins in the context of antigen receptor signaling is by ubiquitin-dependent degradation of autophosphorylated (activated) Syk/ZAP70 kinases. Indeed, previous studies have demonstrated that Syk/ZAP-70 mutants with Y→F substitution of the Cbl binding site impart hyperresponsiveness to TCR, BCR, or FcER1 triggering (Kong et al., 1996; Sada et al., 2000; Zhao and Weiss, 1996), and ZAP70-Y292F knockin mice exhibit reduced TCR downregulation, enhanced proximal TCR signaling, and a higher number of T cells producing interleukin 2 and interferon γ in response to antigen (Magnan et al., 2001). Of note, transfection studies in Jurkat cells led Liu and colleagues to suggest a paradoxical positive role of Cbl-b in TCR signaling (Zhang et al., 1999); this finding remains to be confirmed and is not supported by the phenotype of Cbl-b and Cbl/Cbl-b knockout mice or studies of their T cells.

Cbl proteins readily associate with Src family kinases, including Fyn and Lck, via the following: the Src family kinase SH3 domain interaction with proline-rich sequences of Cbl proteins; the Src family kinase SH2 domain binding to phosphorylation sites on Cbl proteins; and an emerging role of the Cbl TKB domain binding to activation loop phosphorylation site in Src family kinases. A number of transfection studies have shown that interaction of Src family kinases with Cbl proteins leads to their ubiquitin-dependent degradation and inhibition of downstream signaling (Andoniou et al., 2000; Rao et al., 2002b; Sanjay et al., 2001). Notably, the ubiquitinylation of Lck was observed upon CD4/TCR triggering in a normal human T cell clone (Rao et al., 2002b). Furthermore, reduced Lck ubiquitinylation and elevated kinase activity was observed in immortalized T cells from Cbl knockout mice, as was an increased Lck localization to membrane rafts (Hawash et al., 2002; Rao et al., 2002b). The Lck kinase activity was also elevated in anti-CD3-stimulated thymocytes from Cbl knockout mice (Murphy et al., 1998), whereas only marginally higher Lyn phosphorylation was observed upon BCR triggering of Cbl-b knockout B cells (Sohn et al., 2003). Thus, negative regulation of Src family kinases is likely to be an important component of how Cbl proteins exert negative control of antigen receptor signaling. More detailed analyses under conditions of complete lack of Cbl proteins (double knockouts, siRNA) are, however, clearly needed to fully assess the role of Src family kinase regulation by Cbl proteins in antigen receptor signaling.

Ubiquitinylation and Degradation of Downstream Signaling Proteins

As illustrated in Figure 3, Cbl and Cbl-b associate with a large repertoire of signaling proteins other than PTKs (Dikic et al., 2003; Lupher et al., 1999; Miyake et al., 1997; Sawasdikosol et al., 2000; Thien and Langdon, 2001; Tsygankov et al., 2001; Weissman, 2001). Recent studies demonstrate that interaction with and subsequent ubiquitinylation of several of these nonkinase targets provide an additional, and potentially critical, mechanism of negative regulation of antigen receptor signaling by Cbl proteins.

Cbl and Cbl-b associate with Vav, a hematopoietic-restricted Rac/Rho guanine nucleotide exchange factor, upon TCR stimulation. This interaction is thought to be mediated by the Vav SH2 domain binding to Cbl pY700 (Miyake et al., 1997), although Cbl-b association with Vav was shown to require Vav SH3 as well as the SH2 domains (Keane et al., 1995). Moreover, Cbl-b was shown to inhibit the Vav-dependent increase in JNK activity in transfected fibroblasts (Bustelo et al., 1997). A number of prominent biochemical defects in the T cells of Cbl-b$^{-/-}$ mice indicate a critical role of Cbl proteins in the negative regulation of Vav guanine nucleotide exchange factor upon antigen receptor stimulation. Cbl-b-deficient mouse T cells exhibit an elevation in the levels of phosphorylated Vav, Vav activity, and T cell receptor clustering upon receptor stimulation (Bachmaier et al., 2000; Chiang et al., 2000; Krawczyk et al., 2000). Furthermore, Cbl-b deficiency rescued the peripheral T cell proliferation defect in Vav$^{-/-}$ mice and restored the defective T cell receptor clustering and CDC42 activation in Vav$^{-/-}$ T cells (Krawczyk et al., 2000). These studies in T cells are further supported by Cbl-b-mediated inhibition of the oncogenic activity and JNK activation induced by Vav overexpression in fibroblasts (Bustelo et al., 1997). Interestingly, while Vav phosphorylation did not appear to be elevated in anti-IgM-stimulated Cbl-b$^{-/-}$ B cells, Vav-Syk association was more pronounced and longer lasting (Sohn et al., 2003).

Two potential mechanisms of ubiquitin ligase-dependent negative regulation of Vav by Cbl proteins have emerged recently. Phosphorylated Cbl protein, via pY700, can physically associate with Vav (presumably by binding to Vav SH2 domain) (Marengere et al., 1997; Miura-Shimura et al., 2003). This interaction was shown to target Vav for ubiquitinylation, leading to the loss of phosphorylated Vav and inhibition of Vav-mediated NFAT activation; the ubiquitin ligase activity of Cbl was essential for these effects (Miura-Shimura et al., 2003). An indirect mechanism for ubiquitin ligase-mediated

negative regulation of Vav by Cbl proteins has been suggested by recent studies that have demonstrated that Cbl-b targets the p85 subunit of PI 3-kinase for ubiquitinylation but apparently not for degradation; instead, the PI 3-kinase ubiquitinylation was associated with a reduction in its recruitment to the TCR ζ chain and CD28 (Fang and Liu, 2001). The authors suggested that the reduced PI 3-kinase recruitment and consequent reduction in the PI 3-kinase product phosphatidylinositol-(3,4,5) trisphosphate at the membrane leads to a lower level of Vav activation mediated by its plextrin homology domain (which binds to the PI 3-kinase product); consistent with this model, PI 3-kinase inhibitors abrogated the hyperphosphorylation of Vav and hyperproliferation and higher IL2 production observed in unstimulated or anti-CD3-stimulated Cbl-b$^{-/-}$ T cells. It is likely that the two mechanisms discussed above are not mutually exclusive and function together to effectively control the level of Vav activity induced by antigen receptor stimulation. Of note, however, Cbl/Cbl-b double knockout T cells show no apparent evidence of Vav hyperactivity, despite massive hyperresponsiveness to T cell receptor triggering (Naramura et al., 2002), suggesting that more complex biochemical mechanisms mediate the regulation of Vav exchange activity by Cbl ubiquitin ligases.

While indirect regulation of Vav function is one of the potential consequences of PI 3-kinase ubiquitinylation by Cbl family proteins, it is quite likely that other functions of PI 3-kinase are also affected. It is notable that the proportion of cellular PI 3-kinase activity that becomes associated with Cbl upon antigen receptor stimulation is substantial (Fukazawa et al., 1995; Kim et al., 1995; Panchamoorthy et al., 1996); thus, direct negative regulation of PI 3-kinase signaling by Cbl proteins is likely to be of considerable significance. Consistent with this idea, the level of phosphorylated AKT, a crucial downstream effector of PI 3-kinase, is increased in Cbl-b knockout T cells (Fang and Liu, 2001).

Antigen receptor stimulation has been shown to induce the interaction of phosphorylated Cbl or Cbl-b with the SH2 and SH3 domain-containing adaptor proteins of the Crk family (Crk-I and Crk-II are alternatively spliced products of a single gene while Crk-L is a distinct Crk-like gene product) (Miyake et al., 1997; Tsygankov et al., 2001). While the SH2 domain of Crk proteins interacts with Cbl proteins (the binding site in Cbl corresponds to pY774), the SH3 domains of these adaptors interact with C3G, a guanine nucleotide exchange factor for Ras-like small G protein Rap1, promoting the assembly of a ternary complex of Cbl, CrkL, and C3G upon antigen receptor stimulation (Boussiotis et al., 1997; Ingham et al., 1996; Reedquist et al., 1996; Smit et al., 1996). While some studies initially suggested that the Cbl-Crk-C3G complexes may stimulate the Rap1 pathway (Boussiotis et al., 1997; Schmitt and Stork, 2002), these findings have not been confirmed, and recent studies strongly suggest that Cbl and Cbl-b function as negative regulators of Rap1 activation. Compared to wild-type cells, the Cbl$^{-/-}$ thymocytes and Cbl-b$^{-/-}$ peripheral T cells showed higher levels of CrkL-C3G complexes and increased Rap1 activity upon TCR engagement (Shao et al., 2003; Zhang et al., 2003). These findings are of great interest as recent studies demonstrate that Rap1 does not play a negative role in T cells as suggested by earlier analyses (Boussiotis et al., 1997; Schmitt and Stork, 2002) but rather plays a positive role in antigen receptor signaling, as shown by enhanced positive selection of T cells in transgenic mice expressing an activated Rap1 in T cells (Sebzda et al., 2002). Transgenic T cells expressing the activated Rap1 show increased LFA-1 integrin avidity, consistent with the recently assigned role for Rap1 in enhancing the avidity of integrins through inside-out signaling (Bos et al., 2001). Indeed, Cbl$^{-/-}$ thymocytes and Cbl-b$^{-/-}$ peripheral T cells showed unchanged levels of LFA-1 but increased binding to LFA-a ligand ICAM-1 (Shao et al., 2003; Zhang et al., 2003). Thus, Cbl family proteins, by reducing the levels of antigen receptor-induced Rap1 G protein activity, are likely to interfere with the stability of the immunological synapse, which requires integrin-mediated interactions between an activated T cell and an APC. As discussed below in the context of anergic T cell signaling, this indeed appears to be the case.

As further studies define the precise fate of other signaling protein complexes involving Cbl proteins, it is likely that additional biochemical pathways used by Cbl proteins to negatively regulate antigen receptor signals will emerge.

Ubiquitin-Dependent Downregulation of Activated Antigen Receptors via Lysosomal Targeting

Recent analyses have firmly established that recruitment of Cbl family proteins to activated growth factor receptor tyrosine kinases mediates receptor ubiquitinylation, a modification crucial for sorting of the internalized receptors to lysosomes (Dikic et al., 2003; Lupher et al., 1999; Miyake et al., 1997; Rao et al., 2002a; Sawasdikosol et al., 2000; Thien and Langdon, 2001; Tsygankov et al., 2001; Weissman, 2001). This reflects an evolutionarily conserved function of the monoubiquitin species as recognition signals for Endosomal Sorting Complex Required for Transport (ESCRT) proteins, which play an essential role in sorting ubiquitinylated receptors into the interior of the invaginating vesicles of the multivesicular body (MVB) (Raiborg et al., 2003) (Figure 4). Upon maturation or fusion of the MVB to lysosomes, the receptors within the internal vesicles are released into the lysosomal hydrolase-rich acidic environment for degradation. In the absence of ubiquitin modification, the internalized receptors escape the lysosomal sorting and recycle instead (Dikic et al., 2003; Lupher et al., 1999; Miyake et al., 1997; Raiborg et al., 2003; Rao et al., 2002a; Sawasdikosol et al., 2000; Thien and Langdon, 2001; Tsygankov et al., 2001; Weissman, 2001) (Figure 4). Identification of a crucial role of Cbl ubiquitin ligases in receptor tyrosine kinase sorting to the lysosome has therefore led to the notion that these proteins also serve to downregulate activated antigen receptors via ubiquitin-dependent lysosomal sorting (Lupher et al., 1999; Rao et al., 2002a). The transmembrane signaling chains of antigen receptors, such as TCR ζ, are known to undergo ubiquitinylation upon receptor engagement (Alcover and Alarcon, 2000; Cenciarelli et al., 1996). Furthermore, stimulus-dependent internalization of antigen receptors and their degradation in lysosomes has been well documented (Alcover and Alarcon, 2000; Jang and Gu, 2003; Valitutti et al., 1997). However, whether antigen receptor ubiquitinylation is

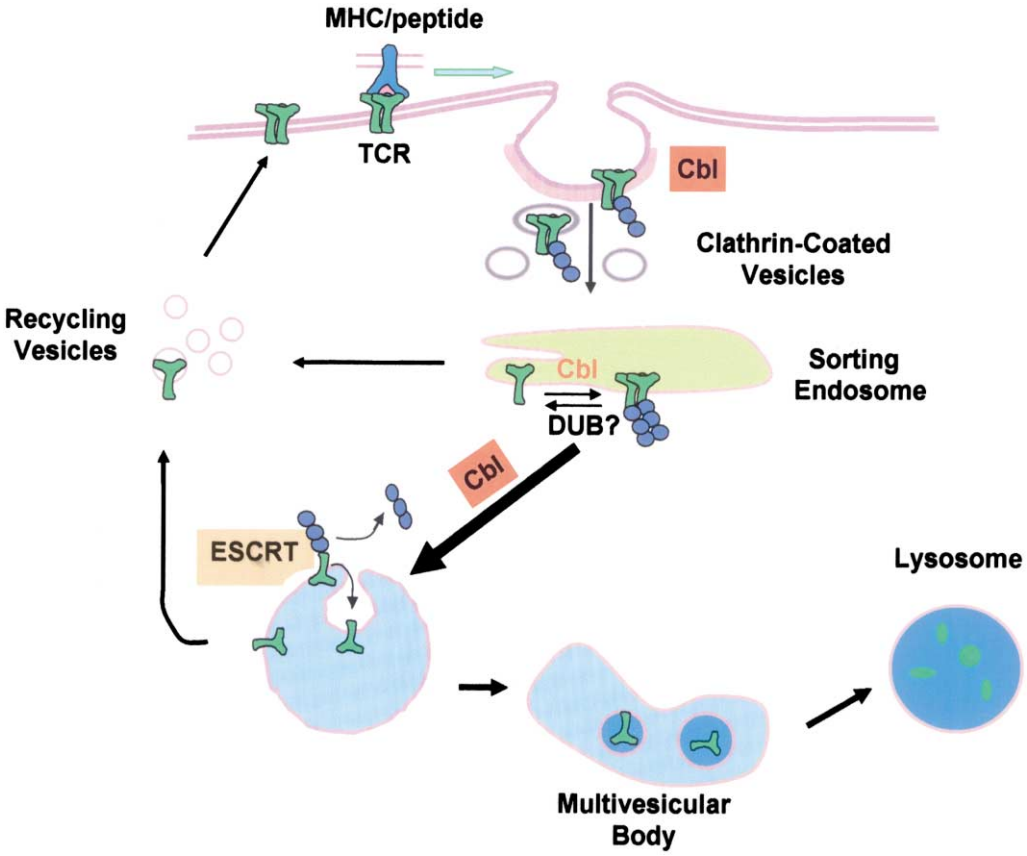

Figure 4. Negative Regulation of Antigen Receptor Signaling through Ubiquitin-Mediated Downregulation of Activated Receptors

Ligand-induced activation of receptors induces their internalization through clathrin-coated vesicles. Ubiquitin modification of the signaling subunits of antigen receptors by Cbl family or other E3s is recognized by ESCRT (Endosomal Sorting Complex Required for Transport) proteins on the multivesicular body (MVB) compartment of the endocytic pathway. The ubiquitinylated receptors are sorted into the inner vesicles of the MVB and are subsequently degraded in the lysosomes, resulting in receptor downregulation and termination of receptor signaling. The nonubiquitinylated or deubiquitinylated receptors through deubiquitinylation enzymes (DUBs) follow the recycling endosomal pathway, escape degradation, and reinitiate signaling upon ligand binding. Increased abundance of E3s in anergic T cells would favor the degradative versus the recycling fate of activated antigen receptors.

mediated by Cbl family proteins and whether the receptor downregulation via lysosomal degradation is an important mechanism to control the intensity of antigen receptor signals were not apparent until recently. In fact, there is a strong correlation of T cell receptor internalization by peptide-MHC with T cell activation (Bachmann et al., 1997; Itoh et al., 1999; Valitutti et al., 1995). Recent analyses, however, strongly support the crucial role of Cbl family ubiquitin ligases in the downregulation of antigen receptors, and defective downregulation of the TCR correlated with enhanced TCR signaling. A major defect in T cells with deletion of both Cbl and Cbl-b was a marked reduction in the ligand-dependent downregulation of TCR (Naramura et al., 2002). Notably, minor defects were observed in single knockout T cells as well. While the authors did not directly show that reduced downregulation was associated with a defect in the ubiquitinylation of TCR or associated chains, transfection analyses in Jurkat cells have shown that TCR ζ chain can be targeted for ubiquitinylation by Cbl in a ZAP70-dependent manner (Wang et al., 2001). Cbl was also shown to control the constitutive endocytic degradation of the pre-T cell receptor (Panigada et al., 2002). A synthesis of previous studies on the role of TCR internalization and the recent data on Cbl ubiquitin ligase-mediated lysosomal degradation of receptors suggests that ubiquitin modification of TCR (and other antigen receptor) chains marks them for lysosomal sorting; in the absence of the ubiquitin modification, internalized receptors recycle back to the cell surface for continued engagement by the ligand and repeated cycles of activation (Figure 4). Thus, the lysosomal sorting step, rather than the internalization step, appears to be crucial in the ubiquitin ligase-mediated attenuation of receptor signals. This suggestion is supported by the finding that EGF receptor internalization is unaffected by Cbl deficiency or lack of ubiquitinylation but lysosomal targeting is substantially impaired (Duan et al., 2003).

Role of Cbl and Other Ubiquitin Ligases in Establishing the Program of Lymphocyte Anergy

Induction of lymphocyte anergy, characterized by lack of responsiveness to an otherwise effective antigen, has emerged as a fundamental mechanism for preventing autoimmune responses to self-antigens and may be an important mechanism by which tumors evade immune

surveillance. Biochemical mechanisms of anergy induction have been particularly elucidated in T lymphocytes. Effective T lymphocyte activation requires TCR engagement together with a costimulatory signal, typically received through CD28; TCR engagement in the absence of costimulation leads to anergy (Appleman and Boussiotis, 2003; Schwartz, 2003). A number of experimental protocols, including the stimulation of T cells with calcium ionophores (which activate the calcineurin-dependent NFAT-mediated transcription), also induce T cell anergy and have been particularly useful for examining the signaling pathways that contribute to the establishment of the anergic state.

Based on previous analyses that the anergic phenotype was dominant and dependent on NFAT-mediated transcription, differential display or cDNA microarray technologies were employed to identify the newly induced gene products that contributed to anergy (Anandasabapathy et al., 2003; Davis and Ben-Neriah, 2004; Heissmeyer et al., 2004; Macian et al., 2002). Prominent among the genes that are transcriptionally induced during the establishment of the anergic state are three distinct families of ubiquitin ligases: two RING finger type E3s, Cbl-b and GRAIL; and Itch, a HECT (homologous to E6-associated protein C terminus) domain E3 of the NEDD4 family (Figure 1). Recent studies indicate a crucial role of these proteins in the establishment of anergy.

GRAIL (gene related to anergy in lymphocytes) is a member of a distinct family of RING finger domain-containing E3s that include the *Drosophila* Goliath and the Goliath-related proteins in mammals (Anandasabapathy et al., 2003). This transmembrane protein is constitutively localized in the endosomes and contains an N-terminal protease-associated domain of unknown function that is expected to reside in the lumen of the endosomes, together with coiled-coil and RING finger domains expected to be exposed to the cytosol. The RING finger domain of GRAIL was demonstrated to mediate E3 ubiquitin ligase activity. Notably, while GRAIL is constitutively expressed in certain tissues (such as brain, kidney, and liver), it is nearly undetectable in resting T cells and is induced under anergizing (antigen presented by B7-negative APC) but not activating (antigen presented by B7-expressing APC) conditions in a calcineurin-dependent manner. Overexpression of GRAIL in T cells severely impaired the IL2 production upon antigenic stimulation, and this effect required the ubiquitin ligase function of GRAIL. As the targets of GRAIL-mediated ubiquitinylation remain to be identified, the precise mechanism by which it promotes the anergic state remains to be elucidated. It is notable that the use of inhibitors indicated that a functional endocytic pathway was required for GRAIL-mediated inhibition of IL2 production. Thus, it is possible that, similar to Cbl family ubiquitin ligases, GRAIL regulates the endocytic fate of receptors crucial for full T cell activation. Alternatively, GRAIL may target signaling intermediates localized on the endosomes (Anandasabapathy et al., 2003). Interestingly, GRAIL itself interacts with a regulatory protein Otubain 1, which facilitates its autoubiquitinylation and degradation. In fact, the overexpression of Otubain 1 in naive T cells induced the enhanced proliferation and IL2 production. In contrast, a naturally occurring alternative form of Otubain stabilized GRAIL and enhanced its function. GRAIL and Otubain form a ternary complex with a deubiquitinylating enzyme USP8, suggesting that the function of GRAIL is under positive as well as negative regulatory control (Soares et al., 2004).

Calcineurin-dependent induction of T cell anergy by sustained Ca^{2+} signals (using ionomycin treatment) was shown to be associated with a marked upregulation in the levels of Cbl-b, GRAIL, and the NEDD4 family ubiquitin ligase Itch (Heissmeyer et al., 2004). When the anergic T cells were stimulated through the TCR, a profound loss of PLC-γ1 and PKC-θ through ubiquitin-dependent degradation was observed. Notably, PLC-γ1 served as a substrate for Itch and NEDD4. As proteasome inhibitors did not rescue the PLC-γ1 degradation, and the induction of anergy was associated with increased levels of TSG101 (a component of the ESCRT-1 complex) and monoubiquitinylation of PKC-θ, PLC-γ1 degradation may be a result of lysosomal targeting of the TCR-associated signaling apparatus (Heissmeyer et al., 2004).

Similar to Cbl-b, Itch has been implicated as a negative regulator of T cell activation. Itch-deficient (Itchy) mice show enhanced Th2 cell differentiation and cytokine secretion, a defect linked to Itch-mediated ubiquitinylation and subsequent degradation of JunB and possibly c-Jun (Fang et al., 2002). Given the localization of Itch to endosomes, its interaction with and ubiquitinylation of the endosomal traffic-associated protein endophilin A, and the role of Itch in targeting CXCR4 to the lysosomes (Angers et al., 2004; Marchese et al., 2003), it is likely that Itch participates in the ubiquitin-dependent lysosomal sorting of antigen receptor signaling apparatus. In support of a critical role for the induced ubiquitin ligases in anergy, T cells from mice deficient in Cbl-b or Itch were resistant to anergy induction by ionomycin as well as the associated degradation of PLC-γ1 and PKC-θ (Heissmeyer et al., 2004). Although the studies by Heissmeyer and colleagues concentrated on PLC-γ1, it is likely that increased levels of the E3 proteins target multiple pathways for ubiquitin-dependent degradation via lysosomal as well as proteasomal degradation.

Remarkably, T cells preexposed to anergic stimulation showed a rapid disintegration of the immunological synapse; in contrast, Cbl-b-deficient T cells were mostly protected from such synapse disintegration (Heissmeyer et al., 2004). The anergy-associated immunological synapse disintegration involved a breakdown of the outer LFA-1 ring and aberrant morphology of the central TCR core, and a similar phenotype could be induced by PLC-γ1 inhibitors, suggesting an important role of signaling pathways activated through PLC-γ1 in the stabilization of the immunological synapse. The precise nature of these biochemical pathways remains unknown. Previous studies have suggested that elevated Rap1 GTPase activity, as a result of a constitutive Fyn-Cbl-Crkl-C3G signaling complex, may contribute to T cell anergy by antagonizing Ras effector activation (Boussiotis et al., 1997). However, recent studies have shown that Rap1 GTPase enhances integrin avidity and provides positive signals during T cell development (Bos et al., 2001; Sebzda et al., 2002), and the levels of active Rap1 are elevated in Cbl-deficient thymocytes and Cbl-b-deficient peripheral T cells (Shao et al., 2003;

Zhang et al., 2003). Thus, the disintegration of the peripheral LFA ring of the immunological synapse in anergic T cells may be due, in part, to a reduction in Rap1 activity. In support of this possibility, the TCR-induced Rap1 activation and increased adhesiveness through LFA-1 were recently shown to be PLC-γ1 dependent, very likely due to the involvement of the calcium and diacylglycerol-activated Rap1 exchange factor CalDAG-GEF1 (Katagiri et al., 2004). Furthermore, a Rap1 regulator RAPL associates with LFA-1 and localizes to the immunological synapses upon TCR engagement (Abraham, 2003; Katagiri et al., 2003). However, it has also been argued that increased Rap1 activity reported by some investigators (Boussiotis et al., 1997) may coopt TCR signaling through preactivating integrins and prematurely activating the E3-dependent anergy program (Heissmeyer et al., 2004). In depth biochemical analyses of signaling pathways in anergic versus normal T cells should therefore yield important clues to how ubiquitin ligases control normal antigen receptor signaling and how these processes are altered during lymphocyte anergy. While the studies that have elucidated the connection between anergy and ubiquitin ligases have been carried out in T cells, given the similarities of signal transduction and regulatory pathways downstream of different antigen receptors, these mechanisms may prove to be broadly applicable in immunology.

Conclusion

Studies of the Cbl family of ubiquitin ligases have revealed a novel degradative control of multiple aspects of lymphocyte antigen receptor signaling, which ensures an appropriate level of immune response. Furthermore, a number of additional ubiquitin ligases have been directly linked to the negative regulation of early antigen receptor signaling. Inactivation of the Cbl family and other ubiquitin ligase pathways leads to uncontrolled immune responses, often with frank autoimmunity. Conversely, upregulation of the same ubiquitin ligase pathways has emerged as a key mechanism for the induction of lymphocyte anergy. Further biochemical and cell biological studies of ubiquitin-dependent antigen receptor signaling therefore promise to provide new and important insights into the regulation of normal immune response and autoimmunity. While many of the initial studies have focused on T cells, these mechanisms are likely to extend to structurally and functionally related BCR and Fc receptors. Thus, manipulations directed at antigen receptor-associated ubiquitin ligase pathways should open a new avenue of immunomodulation in autoimmune diseases, allergy, and other immunological diseases.

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